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EVALUATING SPERM DNA FRAGMENTATION INDEX USING TUNEL ASSAY FOR UNEXPLAINED INFERTILITY: ITS ASSOCIATION WITH DEMOGRAPHIC AND LIFESTYLE FACTORS

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Abstract

Infertility has been affecting globally, influenced by both men and women factors. Evaluation of female factor and male factor using traditional semen analysis were carried out on the routine basis. In our study findings, we observed males with normal semen analysis report have abnormal readings of DNA fragmentation index (more than 12%).We assessed the DNA integrity using TUNEL assay by measuring the sperm DFI using flow cytometry. Demographic and lifestyle information including age, smoking habit, alcohol consumption, occupational status, educational profile, clothing type and dietary factors were noted. It was observed that smoking, alcohol and non-vegetarian diet were associated with increased values of DNA fragmentation (>12%). Out of total 110 subjects, 51% were having normal values of DFI whereas 49% of the subjects showed high DFI values. Maximum DFI calculated was 42.5%, which was much higher than the threshold value. The major lifestyle factors discussed in the present study are amongst the potential risk factors that could impair the male fertility. Thus, greater awareness and recognition provide better treatment options for those couples seeking conception.

Keywords: Unexplained Infertility, TUNEL assay, Sperm DNA fragmentation, Semen analysis, lifestyle factors.

Introduction

During the past decades, an increasing trend in male infertility may be observed (1). Because of increase in infertility among males than females it is important to study the male contribution to infertility (2). Semen analysis is regarded as fundamental investigation tool in almost all andrology laboratories but still it cannot differentiate fertile from infertile men. It may be possible that men with normal semen parameters have reduced fertility potential (3). A

significant ratio of couples are being classified as unexplained infertile with normal semen parameters with at least 1 year of unprotected intercourse (4). Influence on fertility rates on males can be because of various factors like lifestyle habits, diet type, stress, smoking, alcohol consumption, etc. (5). It is very important to investigate the various lifestyle factors that in one or the other way affect the reproductive life of the couples. Some studies also observed that wearing of tight underwear impact negatively on the sperm DNA integrity (6). However, comprehension of an additional parameter that enables to assess the sperm DNA damage along with other parameters of the semen analysis will provide a more comprehensive evaluation of the sperm quality. The objective of our present study was to evaluate the association of various lifestyle habits with semen parameters and sperm DNA integrity using TUNEL assay, which will be helpful to understand the cause of idiopathic infertility in unexplained infertile couples.

Materials and methods

Study design and patient population

This cross-sectional study was carried out on 110 participants. It was conducted from March 2019- April 2022 at the IVF center, Department of obstetrics and gynaecology, Adesh Hospital, Bathinda, Punjab. The study was approved by the Institutional Review and Ethical Committee of Adesh University, Bathinda. Minimum 2 days of sexual abstinence was asked to participants prior to the day of investigation.

Inclusion criteria- male partners with normal semen analysis with no surgical history, female factor normal

Exclusion criteria- male partners with abnormal semen parameters, couples with female factor infertility.

Procedure

Semen sample collection was done at the IVF Center in a sample collection room in a sterile, non -toxic plastic container. The collected semen samples were analyzed according to the World Health Organization (WHO) guideline manual, Sixth edition, 2021 for semen analysis within 60 minutes after ejaculation. Macroscopic parameters like volume, pH, liquefaction and viscosity were observed and noted. Microscopic examination was also performed to evaluate the sperm concentration, total sperm number, sperm motility and morphology.

Study participants were interviewed including questions about socioeconomic status, occupation, lifestyle factors (consumption of alcohol, smoking, dietary habits, wearing loose or tight underwear).

Sperm DFI Examination

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate- nick end labeling (TUNEL) assay was the technique used for the assessment of sperm DNA damage. The TUNEL kit utilized in this procedure was the APO-DIRECT^{TM}. This kit labels both single and double DNA breaks and measures the percentage of cells with labeled DNA. The flow cytometry

FacScan (Becton Dickinson) was used to analyse the TUNEL stained sperm cells. The analysis of flow cytometry data was carried out using dedicated software which imply that the DFI histogram was used to precisely determining the percentage of DFI. For the flow cytometry setup and calibration, a reference semen sample was used from the normal donor ejaculate retrieved from the IVF laboratory. The same reference sample was used for the entire study.

12% sperm DFI was used as threshold for our study experiment. This cut-off value was used for the discrimination between healthy semen sample and one that have positive DNA damage.

Results

Demography of study participants

The study participants consisted of 110 men who were attending infertility center for diagnostic purposes. We have come across several interesting findings in our experiment.

The mean age of the men participating in our study was 34.1 ± 3.6 years (Range = 25 – 44) old. As shown in Table 1, the largest age group was 31- 35 years comprising 54.5% of the total number of participants whereas the least age group was 20 -25 years (0.9%).

Age	**Frequency**	**Percentage (%)**
20-25	1	0.9
26-30	14	12.7
31-35	**60**	**54.5**
36-40	29	26.4
41-45	6	5.5
Total	110	100

Table 1 shows age frequency distribution among study participants

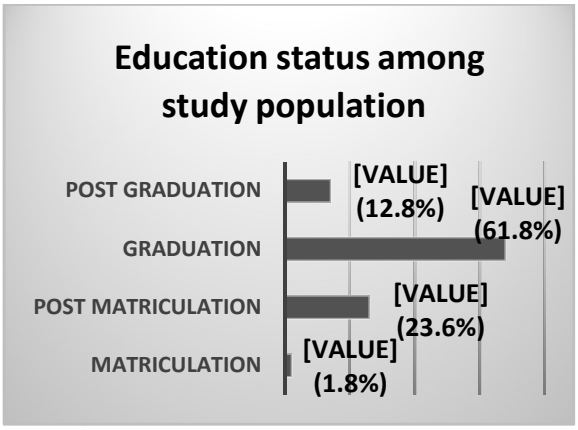

Figure 1 showing education status of the study participants.

Figure 1 depicting the education status of the study subjects, most of them has done graduation that was found to be 61.8%, followed by post- graduation by 23.6 % of the participants while only 1.8 % did only matriculation.

The sexual abstinence range among the study participants before the semen analysis was 2-7 days in this study. Around 36% (that was maximum) of the participants were showing sexual abstinence of 3 days before the study experiment.

Semen parameters among study participants

The various parameters measured in the semen analysis including semen volume, pH, Total sperm count, Sperm concentration, motility and morphology are shown in Table 2. All the semen parametric values were falling in the normal ranges given by World Health Organisation manual, 2021.

Parameters	**Mean ± SD**
Volume (ml)	2.2 ± 0.4
Liquefaction Time (minutes)	23.5 ± 2.8
pH	7.3 ± 0.1
Sperm concentration (millions/ejaculate)	18.3 ± 2.86
Total Sperm Number (millions/ ml)	39.2 ± 3.42
Motility (%)	42.9 ± 3.18
Morphology (%)	4.5 ± 0.94

Table 2 presents mean ± SD values of various parameters semen analysis in the study participants

Table 3: Effect of lifestyle habits on semen parameters

lifestyle parameters	**volume (ml)**	**Sperm concentration (million)**	**Total sperm Count (million)**	**Motility (%)**	**Morphology (%)**
alcoholic	2.2 ± 0.32	17.8 ± 2.51	39.5 ± 3.78	43.4 ± 2.60	4.5 ± 0.91
non-alcoholic	2.1 ± 0.38	18.9 ± 3.15	38.8 ± 2.89	44.5 ± 3.73	4.6 ± 0.98

p-value	0.14	**0.04***	0.28	0.07	0.58
Smoker	2.2 ± 0.32	17.5 ± 2.38	39.4 ± 4.02	43.6 ± 2.75	4.8 ± 1.15
Non- smoker	2.2 ± 0.36	18.5 ± 2.95	39.2 ± 3.25	44.0 ± 3.31	4.5 ± 0.85
p- value	1	0.12	0.8	0.59	0.16
Vegetarian	2.1 ± 0.36	19.9 ± 3.10	38.6 ± 2.93	44.8 ± 3.42	4.5 ± 0.93
Non- vegetarian	2.2 ± 0.33	17.8 ± 2.61	39.6 ± 3.64	43.4 ± 2.95	4.6 ± 0.94
p- value	0.14	**0.0002***	0.14	**0.02***	0.6
Loose	2.2 ± 0.29	18.2 ± 2.25	39.3 ± 2.90	44 ± 2.48	4.5 ± 0.76
Tight	2.2 ± 0.29	18.3 ± 2.25	39.2 ± 2.88	43.9 ± 2.45	4.5 ± 0.75
p- value	1	0.81	0.85	**0.02***	1

* Statistically significant p value < 0.05.

The parameters of the semen analysis were categorized under various lifestyle factors. Significance of association of the semen parameters with living habits of the participants were done on the basis of p-value. Significant results were observed for the semen concentration for alcohol consumption (p= 0.04) and non- vegetarian diet (p= 0.0002), whereas, sperm motility was associated with clothing factor (p = 0.02) and also with non-vegetarian diet (0.02) as shown in table 3

Sperm DFI among study sample

Out of 110 participants, 56 men (51 %) were having normal DFI values (≤ 12%) whereas in 54 subjects (49 %), DFI were found to be abnormal (more than 12%) as shown in figure 2.

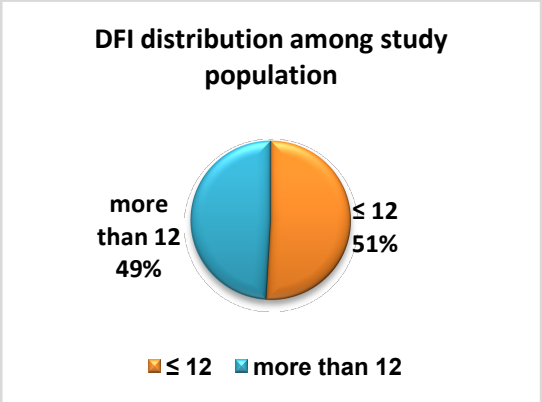

Figure 2 showing sperm DFI distribution among study participants

Out of 110 participants, 36 subjects (33%) were having low values (between 0-5 percent) of sperm DFI whereas 8 men (7.2 %) with high values of DFI were observed (more than 35 %) as shown in figure 3. Maximum value of DFI calculated in our study was 42.5% which was much higher than the normal DFI value.

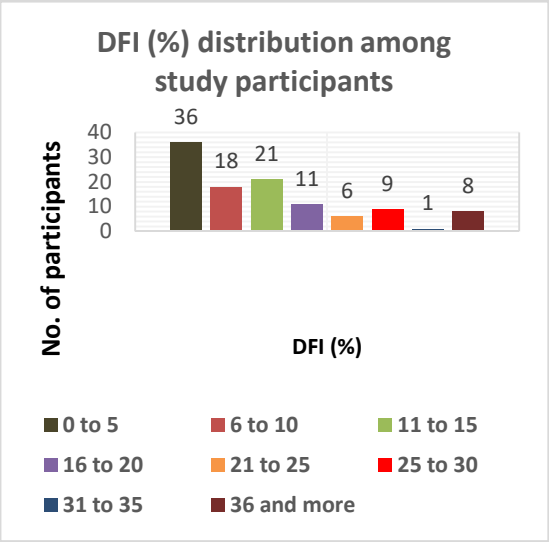

Figure 3 depicts the sperm DFI distribution

It was noted that out of 110 participants, 60 were working in different fields, whereas 28 were having some kind of business and remaining 22 were non – working shown in table 4.

Occupation	**Less than 12%**	**More than 12%**	
Working	29	31	60 (55%)
Business	15	13	28 (25%)
Non-working	12	10	22(20%)
Total	56	54	110

Table 4 showing Occupation profile of the study participants

Lifestyle factors among study participants

85 (77%) men among the total study participants were non- smokers and 23% were having habit of smoking. 17 men were found to have sperm DFI values more than 12%. We examined the influence of cigarette smoking on sperm DNA that smoking was somewhere weakly associated (p-value = 0.04) with sperm DNA integrity as illustrated in table 5.

Factors	**Less than 12%**	**More than 12%**	**Total**	**p- value**
Smoker	8	17	25(23%)	

non-smoker	48	37	85(77%)	
total	56	54	110	**0.0408**

Table 5 showing smoking habit among study participants

Out of total participants, 55% of the men am were having a habit of alcohol consumption and other % were under non-alcoholic category. In our study, it was found that out of 61 who used to consume alcohol, 45 (41%) men were having DFI more than 12%.

Our study found a significant association between alcohol consumption and sperm DFI, as shown in table 6.

Factors	**Less than 12%**	**More than 12%**	**Total**	**P-value**
Alcoholic	16	45	61 (55%)	**<0.00001**
Non-alcoholic	40	9	49 (45%)	
total	56	54	110	

Table 6 presents alcohol consumption of the study participants

Considering the dietary factors including vegetarian and non-vegetarian diet, 64% of the participants were under the category of non-vegetarian and remaining 36% were vegetarians. 42% non-vegetarian participants were showing DFI value above 12%. There was a strong association between diet and DFI values of the study participants as shown in table 7.

Factors	**Less than 12%**	**More than 12%**	**Total**	**P-value**
Vegetarian	32	8	40 (36%)	**<0.00001**
Non-vegetarian	24	46	70 (64%)	
total	56	54	110	

Table 7 depicts dietary habit of the study participants

Among the study participants, 53% of the men wore loose underwear while 47% used to wear tight one. Out of 110 subjects, 28 (25%) men were having high values of sperm DFI and these men were having a habit of wearing tight underwear. Table 8 shows no association was found between clothing types with the sperm DFI value in our study subjects.

clothing	Less than 12%	More than 12%	Total	P-value
loose	32	26	58(53%)	
tight	24	28	52(47%)	**0.445**
total	56	54	110	

Table 8 showing clothing type of the participants

Discussion

Deterioration in the fertility rates among couples globally postulated the need to understand how the advanced living style habits have contributed to this phenomenon. We examined the influence of various lifestyle factors like smoking, dietary habit, clothing and alcohol consumption on traditional semen analysis values as well on the sperm DNA.

In our study, maximum participant's age was ranging from 31 – 35 years and the mean age was found to be 34 years. It was also observed that 62% of the participants did graduations and 55% among the total study subjects were working in different occupational fields.

In this study, we examined the association of lifestyle factors with the traditional semen analysis parameters and results showed significant association of semen concentration with the alcohol consumption and non-vegetarian diet, also sperm motility was associated with tight clothing and non-vegetarian diet. In some previous studies that also reported that habit of alcohol consumption reduced the sperm quality (7, 8). This is in contrast to a study that found no significant differences in sperm parameters of participants who consumed alcohol and those who did not (9).

In the present study, it was observed that wearing tight underwear was associated with negative impact on sperm motility, explaining to this fact, a previous study stated that wearing tight underwear elevated the scrotal temperature causing harmful effect to sperm quality (10). In our study, it was also found that non-vegetarian diet was significantly associated with the sperm concentration. On the other side, in some studies no association was noted among meat intake and the semen parameters (11, 12).

Our results found that out of 110 subjects, 56 men (51%) were having DFI value less than or equal to 12% and remaining 54 (49%) subjects have high DFI (more than 12%). Somewhat similar results were seen in some studies regarding the DFI percentages (13, 14, 15). Moreover, in our study, 8.2% of the total study participants have higher DFI (more than 30%). Almost similar percentage (8.4%) were observed in a previous study with increased DFI (>30%) (14).

As per our study, 60 men (55%) were working by profession in different fields and out of which 31 were having DFI more than 12%. These increased values can be because of work stress and prolonged sitting hours. One of the study investigated a positive association between occupational stress and percentage of sperm DNA defects (16).

There was a significant association found between smoking habit and sperm DFI (p= 0.04) in our findings. Results are in agreement with a previous study which shows smoking group exhibited a significantly higher DFI rate, compared with the non-smoking group (17).

There was a significant association observed between the alcohol consumption and the raised DFI value (p< 0.00001). The process of spermatogenesis appear to gradually decline with increasing level of alcohol consumption (18). On the contrary, no association was found between consuming alcohol and DNA damage (19).

Among dietary factors, non-vegetarian diet showed significant association with the increased levels of DFI. Another study also defined a typical western diet contains red and processed meat was negatively associated with sperm morphology (20). Our findings also concentrated upon clothing factor including loose and tight underwear, which shows no association with DFI (P=0.45). On the other hand, some studies suggested that wearing loose underwear or boxers was associated with lower risk of sperm DNA damage and sperm neck abnormalities (21, 22).

With the substantial increase in research related to sperm DNA fragmentation, several guidelines for sperm DNA fragmentation (SDF) testing were launched for clinical practices (23). This would expand the knowledge for investigating the male partner more deeply for explaining the unknown factors behind the unexplained infertility.

Conclusion

Knowledge of the factors that influence sperm quality at the DNA level can contribute to informed decisions on effective management of explaining the unexplained infertility. Andrologists and reproductive specialists can suggest necessary lifestyle changes and assist the patients towards fertility treatment methods that can offer the best chance for success. Awareness and recognition of the possible impact of risk factors present in our daily lifestyle is crucial amongst couple seeking conception. Therefore, the unexplained infertile couples may benefit from early treatment and clinical interventions to achieve a successful reproductive life.

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